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RECORDS OF *p*H CHANGES DURING ENZYME REACTIONS AND KINETIC STUDIES WITH YEAST HEXOKINASE

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THE solution of problems of enzyme kinetics and of the control of chemical pathways of metabolism requires an ever-increasing number of methods for the continuous recording of physical quantities which help to monitor concentration changes over widely different time scales. With the aid of such methods it is becoming possible to analyse the kinetic behaviour of enzyme reactions in complex biological systems with greater resolution and to detect transient changes in the behaviour of such systems.

In studies of the kinetics and mechanisms of isolated enzymes, methods for the continuous recording of the progress of the reaction without the need to take samples have many obvious advantages, truly initial rates are more easily obtained, changes in the course of the reaction are readily detected and studies over a wide range of rates can be performed. The use of rapid reaction and recording methods has two major applications. First, they enable one to observe transient changes characterizing individual steps of an enzyme reaction either by spectral[1] or kinetic[2] analysis. Secondly, such methods allow one to examine the overall reactions at the high concentrations at which enzymes often occur locally in cells and at concentrations similar to those used for the study of changes in protein aggregation or similar alterations in configuration. The liberation or disappearance of hydrogen ions have been widely used as a means of following hydrolytic and group transfer reactions, the classical method for following ester hydrolysis by titration to a constant *p*H being a good example. For very rapid reactions the spectrophotometric observation of indicator colours initiated by Brinkman, Margaria and Roughton[3] and applied to a variety of systems by Gutfreund[2] offers the simplest procedure for following *p*H changes. For moderately slow reactions the various commercial automatic titrators offer convenient means for the collection of kinetic data.

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With the advent of a wide range of high-impedance amplifiers it has become possible to record *p*H changes directly with an accuracy of 0·001 *p*H unit and initial rates of enzyme reactions can be determined under conditions which involve a total *p*H change of less than 0·05. Such a technique permits moderately rapid recording of enzyme reactions by following *p*H changes. In this paper we shall first demonstrate the time and concentration resolution of the method. We shall further demonstrate the application of this method to the recording of complex reactions of ATP with mitochondria. Some experiments on the use of *p*H records for the study of phosphorylation[4] and endogenous ATPase activity in mitochondria[5] have been reported recently. This method is likely to be of considerable importance for kinetic studies of different ATP hydrolysing systems and our more detailed experiences should be useful. The ratio of product formation to $[H^+]$ liberated during ATP hydrolysis or phosphate transfer by kinases is dependent on the *p*H of the reaction mixture. We have used the *p*H recording technique to determine this ratio for the hexokinase reaction as a function of *p*H and have used this information for a study of some features of the kinetics of yeast hexokinase.

Enzyme preparations and substrates. Trypsin was a twice crystallized sample from Servac Laboratories, Colnbrook, Bucks. Yeast hexokinase was a suspension of crystals in ammonium sulphate obtained from Boehringer and Soehne, Mannheim, Germany. This enzyme preparation phosphorylated 140 μmoles glucose/min/mg enzyme at 25° at optimum glucose, ATP and Mg^{2+} concentration and *p*H 7·5. This specific activity is considerably lower than the highest which can be obtained (approx. 300 μmoles/min/mg), but the enzyme preparation used had the advantage of great stability; 1 mg was dissolved in 10 ml. of water and stored in the cold room. Frequent assay showed that 92 per cent of the activity was retained after two weeks storage. Various column purifications readily give some increase in specific activity with concomitant loss in stability. For all the kinetic investigations described here stock solutions containing 0·1 mg enzyme/ml. were prepared and reactions were initiated by addition of 0·2 ml. enzyme stock to 5 ml. appropriate reaction mixture.

Mitochondria were prepared from guinea pig livers by the method of Schneider[6] and used within 5 h of preparation for experiments involving intact mitochondria.

N-benzoyl-L-arginine ethyl ester hydrochloride was prepared and analysed by Hammond and Gutfreund[7]. Adenosine triphosphate $2Na4H_2O$ was obtained from Sigma Chemical Co., St. Louis, United States, and was found to be better than 98 per cent pure. 'AnalaR' reagents and glass distilled water were used to make up the other components of the reaction mixtures.

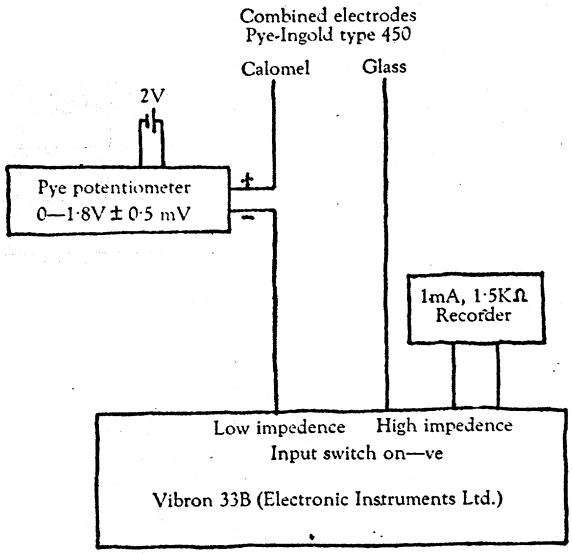

Fig. 1. Schematic representation of circuit for *p*H recording

*Method of recording p*H *changes.* Fig. 1 is a schematic drawing of the arrangement of instruments used for recording changes in mV due to change of *p*H. The combined calomel and glass electrode (Ingold type 450, Pye Instruments, Ltd., Cambridge) was immersed in a 'Perspex' reaction chamber mounted on a magnetic stirrer in a water-bath. Unless otherwise stated reactions were carried out at 25°. The electrodes were standardized[8] with a solution 0·1 M KH_2PO_4, 0·1 M Na_2HPO_4, *p*H 6·671 which is approximately in the centre of the *p*H-range used. The accuracy of absolute *p*H measurements of the reaction mixtures as determined with the instruments specified in Fig. 1 is likely to be of the order of 0·01 *p*H unit (0·59 mV). The stability of the equipment permits the measurement of *p*H changes with an accuracy of 0·001 *p*H unit. Our method of calibration of individual experiments did not rely on any absolute accuracy in terms of *p*H or mV but only on stability and reversibility of the equipment.

Our method of carrying out and interpreting a kinetic experiment involving the liberation of hydrogen ions is best illustrated with the record given in Fig. 2. After the electrode was calibrated by taking a reading in mV with the standard phosphate buffer, the reaction mixture (5 ml. of triethanolamine hydrochloride mM, potassium chloride 0·1 M and *N*-benzoyl L-arginine ethyl ester mM) was pipetted into the reaction vessel. An 'Agla' microsyringe containing 0·1 M sodium hydroxide was used to

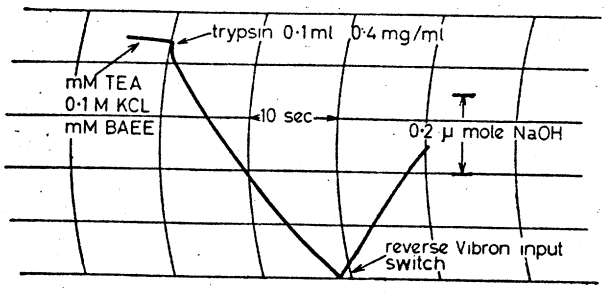

Fig. 2

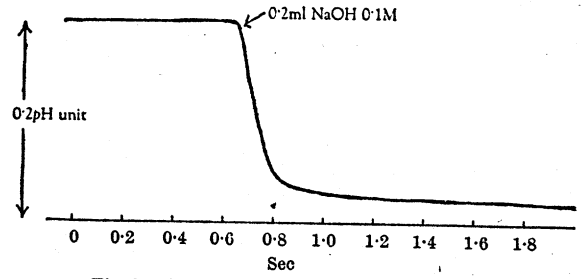

Fig. 3. Oscilloscope record of rapid *p*H change

adjust the *p*H of the solution to 7·9. The last two or three additions of alkali were always used as calibrations in terms of μmoles/mV and these calibrations were used to calculate the time course of hydrogen ion production from the record of change in mV. At the point indicated in Fig. 2, 0·1 ml. of solution containing 0·04 mg trypsin was added and a linear record of mV against time is obtained for the initial rate of hydrolysis of the ester. This was a relatively rapid and sensitive run. The response time of the equipment, including the speed of addition and mixing of enzyme to start the reaction, was tested as follows. The output of the 'Vibron' millivoltmeter was connected to an oscilloscope across an adjustable 5-kΩ resistance and a photograph of the oscilloscope was taken during the addition of alkali (0·2 ml.) to buffer (5 ml.) as indicated in Fig. 3. This record indicates that by the simple procedure of adding enzyme from a 0·2 ml. pipette manually with a teat, observation on a reaction could be made within 0·2 sec from the start of such an addition.

Fig. 4 illustrates the application of the method to studies of ATPase in complex systems. The record shows *p*H changes during the hydrolysis of ATP by guinea pig liver mitochondria on stimulation with DNP. The effect of succinate oxidation on the ATPase activity of intact mitochondria is also demonstrated. A comparison of the

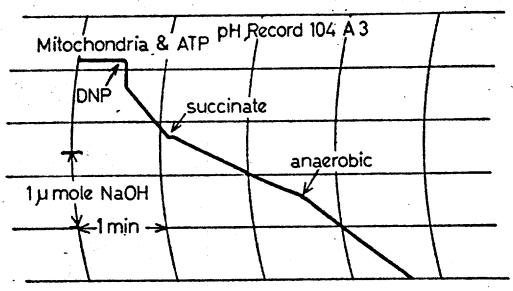

Fig. 4

higher buffer concentration in this experiment as compared with that illustrated in Fig. 2 shows that the ATPase run was deliberately carried out at low sensitivity and high concentration of mitochondria so as to enable us to demonstrate the effect of respiration and the attainment of anaerobiosis. Control experiments showed that under the conditions of this experiment in the absence of ATP, the addition of DNP and/or succinate causes no significant *p*H change.

Studies of the hexokinase reaction. The *p*H dependence of the proton yield of the hexokinase reaction. During the hydrolysis of ATP to ADP and inorganic phosphate or the phosphate transfer from ATP to glucose to form ADP and glucose-6-phosphate, the ratio of hydrogen ions liberated to ATP reacted is *p*H dependent. This has been discussed in some detail by Alberty, Smith and Bock[9]. In alkaline solutions the ratio becomes unity; in acidic solutions it approaches zero. A plot of the ratio against *p*H resembles a titration curve and its precise form is a function of the ionization constants of all reactants and products. For the interpretation of kinetic data in terms of the rate of liberation of hydrogen ions in solutions below about *p*H 7·6 precise curves for the ratio at defined ionic strength and composition are required. Fig. 5 gives the curve obtained for the conditions used in our studies of the *p*H dependence of the hexokinase reaction. The three sets of points represent three series of experiments with different stock solutions of ATP. Excess glucose and ATP were treated with hexokinase at different *p*H, allowed to react to completion and titrated back to the initial *p*H with standard NaOH solution. Experiments on the kinetic behaviour of hexokinase in solutions of different composition were carried out at *p*H 7·5 or above, when corrections become small. The effect of temperature on the ratio is likely to be small since the heat of ionization of phosphate groups is very small.

*p*H *dependence of the hexokinase reaction.* Previous investigations of the *p*H dependence of the hexokinase catalysed reaction by Sols, de la Fuente, Villar-Palasi and Asensio[10] and Kaji, Trayser and Colowick[11] were followed

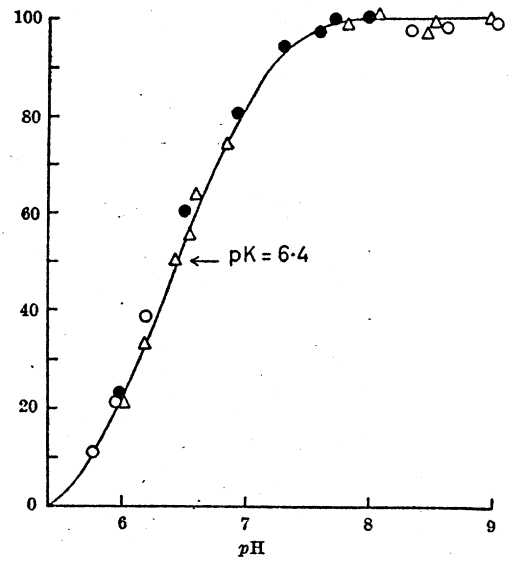

Fig. 5. Percentage H^+ yield from hexokinase reaction—0·9 mM MgATP 0·19 M potassium chloride and buffer

either by the analysis of samples taken from the reaction mixture or by continuous estimation of glucose-6-phosphate by the reduction of NADP in the presence of glucose-6-phosphate dehydrogenase. Such complex methods are liable to much random error, as well as to systematic error as the reaction mixture or other conditions of the experiments change. The two curves presented in Fig. 6 give a simpler function of reaction velocity against *p*H than has been found in the previous investigations. The points above *p*H 8 are not included in the curve because irreversible inactivation in alkaline solutions would make their interpretation difficult. A comparison of the two sets of data obtained at 0·87 mM and 5·19 mM respectively, as well as Michaelis constant determinations at *p*H 6·0 and 7·4, indicate that *p*H influences the apparent Michaelis constant of ATP. This can be due to a variety of phenomena to be discussed later on.

Role of Mg^{2+} *in the hexokinase reaction.* The reaction has an absolute requirement for some divalent ion. This could either be due to the need for such ions in ATP enzyme combination or to their essential role in the subsequent catalytic phosphate transfer. The experiments recorded in Fig. 7 were designed to distinguish between these two effects. If Mg^{2+} were required for ATP enzyme combination the reaction velocity at low Mg^{2+} concentration should be independent of ATP concentration. In contrast to this our experimental results indicate that when the concentration of Mg^{2+} is well below optimal

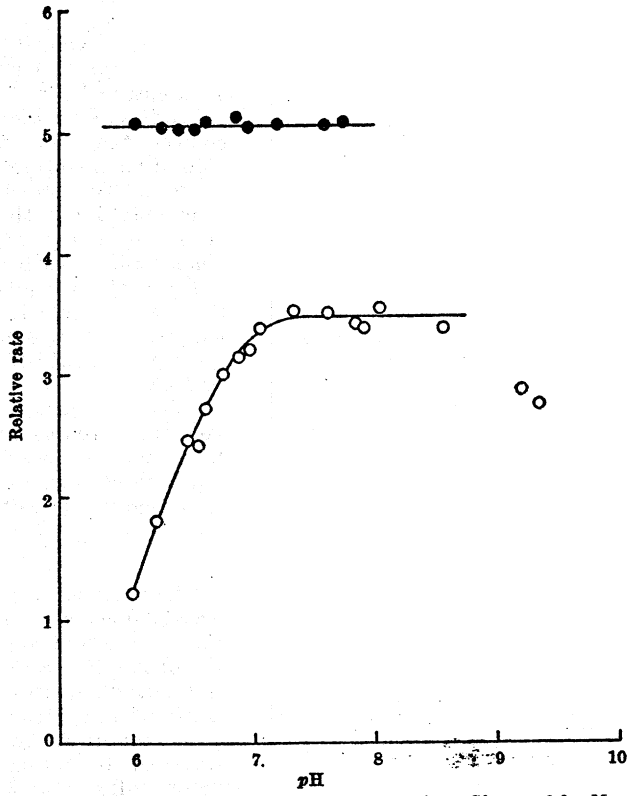

Fig. 6. *p*H dependence of the hexokinase reaction. Glucose, 8·8 mM; ○, ATP = 0·87 mM; ●, ATP = 5·19 mM

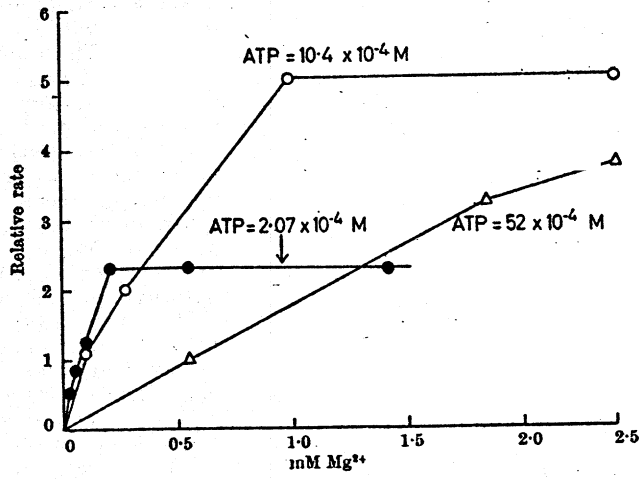

Fig. 7. Hexokinase: effect of Mg^{2+} at different ATP concentrations

the reaction velocity is inversely proportional to the ATP concentration. A probable explanation for these findings is discussed later.

Studies of the effect of Be^{2+} on the hexokinase reaction showed that at concentrations equivalent to ATP the rate of the reaction is 33 per cent of that in the absence of Be^{2+}. The percentage inhibition on addition of beryllium chloride is the same whether magnesium chloride is present at equivalent concentration or only 1/7 of the concentration of beryllium chloride.

Effect of temperature on the reaction. A comparison of the reaction velocity at 25° and 35° showed that at *p*H 6 the rate was increased by a factor of 2·7 at the higher temperature, while at *p*H 7·4 the increase was by a factor of 1·65.

Effects of ionic strength. No significant changes in velocity were observed on omission of potassium chloride from the reaction mixture. The use of solutions of high ionic strength is, however, advantageous for stable records from the glass electrode.

Discussion

The method used in the experiments described in this article does not involve any original principle. It is, however, useful to present a description of the procedures for obtaining accurate *p*H records and calibrating them in terms of the rate of hydrogen ion production or disappearance in a variety of systems of different complexities. It is important to show that meaningful and accurate data can be obtained by a simple method which allows one to follow reaction rates in terms of *p*H changes over a range of time scales from fractions of seconds to several minutes, without any additions and—except for very fast reactions—without stirring during the progress of the reaction. The application of direct *p*H records to the study of ATPase in a wide variety of physiological systems should be possible. The rapid response time and sensitivity of the method allows one to follow the very early stages of the reaction and transient changes in the course of events such as illustrated in Fig. 4. If results obtained by the *p*H recording method and by analysis of free inorganic phosphate are compared questions such as those asked by Wheeler and Whittam[12] about the possible existence of phosphorylated intermediates during the hydrolysis of ATP might be answered.

The relation between the *p*H of the reaction and the ratio $[H^+]$ liberated/[product] is used in this article in a purely operational manner for the interpretation of the experiments on the hexokinase catalysed reaction. For different reactions of ATP a curve such as that given in Fig. 5 has to be obtained and its precise form will depend on the ionization constants of the reactants and product in a medium of the particular ionic composition used for

the experiments. Fortunately correction factors become small under all conditions when reactions involving phosphate transfer from ATP are studied at *p*H 7·5 or above.

The *p*H dependence of the velocity of the hexokinase catalysed reaction cannot be interpreted easily in terms of ionizable groups on the catalytic or binding site of the enzyme. Even in the case of proteolytic enzymes, where such characterization was successful, many precautions have to be taken[13]. The reactions of hexokinase are complicated, in addition, by a number of *p*H dependent changes of both enzyme and substrate. Over the range of *p*H studied by us and by Kaji *et al*[11] and Sols *et al*.[10] ATP will be distributed in varying proportions[14] among the following ionic species $ATPMg^{2-}$, $ATPHMg^{-}$, $ATPH^{3-}$ with negligible amounts of $ATPMg_2$ when the total concentration of ATP was equal to the total concentration of Mg^{2+}. It is not yet known which of these forms of ATP is a substrate, inhibitor or non-reactive species for hexokinase, though some information about the role of Mg^{2+} will be discussed here. It is also known that the hexokinase molecule undergoes aggregation reactions which depend on *p*H as well as a number of other factors[15]. We have found (Andrews, Hammond and Gutfreund, unpublished information) that the gel-filtration method of Andrews[16] applied to hexokinase solutions indicates enzyme aggregation between *p*H 7–6, even at the dilute protein concentrations used for enzyme assays. The *p*H independence of the reaction at high concentrations of $ATPMg^{2-}$ would be consistent with the type of active site postulated by Watts and Rabin[17] for creatine phosphokinase. Taking int ac count our own kinetic results and Barnard and Ramel's[18] experiments on the temperature dependence of the reactivity of essential —SH groups of the enzyme, one can make a tentative proposal for the reaction mechanism. It is known that a substituted chemical enzyme–glucose or enzyme–phosphate compound does not occur as an intermediate[19], but it is likely that a compulsory sequence of events involves a reaction of the enzyme with glucose to make the active site reactive for phosphate transfer from ATP (see Hammes and Kochavi[20], and Hammes, private communication). This type of mechanism was predicted for hexokinase by the induced fit theory of Koshland[21] to explain lack of reactivity of the enzyme with ATP and water in the absence of glucose.

Our data of the Mg^{2+} concentration dependence of the hexokinase reaction at various concentrations of ATP indicate that Mg^{2+} is not required for the combination of ATP with the enzyme but for the subsequent phosphate transfer reaction. ATP^{4-} acts as a competitive inhibitor for $ATPMg^{2-}$ in the enzyme —ATP combination. This is shown by the fact that the reaction velocity at equivalent $ATPMg^{2-}$ concentration is inversely proportional to the

concomitant concentration of ATP^{4-}. This is in agreement with the studies of the effect of Mg^{2+} and Ca^{2+} on the hexokinase reaction by Hammes and Kochavi[22] and it gives an interesting lead for further investigations into the mechanisms of a number of enzyme systems with an absolute requirement for divalent ions.

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